

as the half-maximally stained one.

Intensity of immunohistochemical stainability in each magnocellular neuron was scored according to the following criteria: not stained, weakly stained and heavily stained, by consulting to the arbitrarily selected standard sections. Immunoreactivity was shown by the number of immunoreactive (ir) neurons and the percentage of heavily stained neurons in each of the SON and the PVN. In addition, after depicting the outline of individual ir-neurons with a camera lucida, their cellular areas were determined with a tablet digitizer-microcomputer system.

RESULTS

Autoradiographic signals of the AVP-NP and OXT-NP probes were densely localized over the

magnocellular neurons in the SON, the PVN and several accessory magnocellular nuclei, as was previously reported [9]. The distribution of AVP-NP and OXT-NP probes coincided well with the immunohistochemical localization of AVP and OXT, respectively. The ventral region of the SON and the dorsolateral region of the PVN were predominantly composed of ir-AVP neurons. The AVP-NP probe was localized in these regions (Fig. 2). While, the dorsal region of the SON and the ventromedial region of the PVN were rich in ir-OXT neurons. This distributional pattern coincided well with the localization of the OXT-NP probe (Fig. 3).

AVP neurons

The density of autoradiographic signals representing the localization of the AVP mRNA was

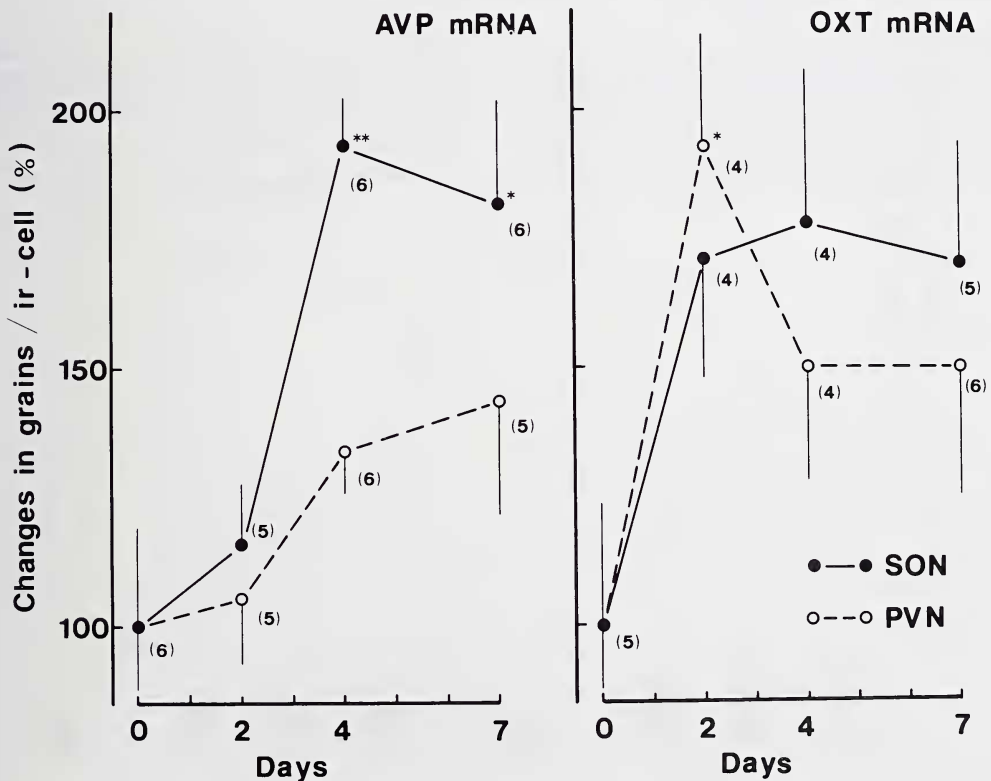


FIG. 4. Effects of sodium loading on the AVP and OXT mRNA levels in the SON and the PVN. The mRNA levels are expressed relatively as percent changes to the level of day 0. The numbers of silver grains/ir-cell on day 0 are: AVP mRNA in the SON, 122.2 ± 13.2 ; AVP mRNA in the PVN, 177.6 ± 13.8 ; OXT mRNA in the SON, 85.9 ± 9.8 ; OXT mRNA in the PVN, 122.4 ± 10.1 . Each point represents the mean \pm S.E. The number of animals is given in parentheses. *, $p < 0.05$; **, $p < 0.01$; by the t-test compared to day 0.

markedly increased in the SON by the sodium loading. The increase became statistically significant after day 4 of the treatment (Figs. 2 and 4). On the other hand, the increase in the hybridization signals in the PVN was much smaller than that in the SON, and was not statistically significant during the treatment (Fig. 4).

The number of ir-AVP cells was decreased in the PVN on day 7, while the percentage of heavily stained cells was significantly reduced in the SON (Figs. 2 and 5). Significant hypertrophy of ir-AVP neurons was observed after day 2 of the treatment in both the SON and the PVN (Figs. 2 and 7). The hypertrophy of supraoptic neurons was more conspicuous than that of paraventricular neurons.

The density of silver grains in the suprachiasmatic nucleus which also includes ir-AVP was only slightly higher than the background level, and was not changed noticeably by the sodium loading.

OXT neurons

The density of hybridization signals for the OXT mRNA was drastically increased and attained to peak levels in both the SON and the PVN on day 2 of the sodium loading. The increase in the PVN

was statistically significant (Figs. 3 and 4). The signal levels in the SON were maintained around the peak level, while those in the PVN were somewhat decreased after day 4 (Fig. 4).

The numbers of stained cells and the percentages of heavily stained cells were not significantly changed in both the SON and the PVN (Figs. 3 and 6). The number of stained cells was slightly increased on day 7. On the contrary, the percentage of heavily stained cells was gradually decreased, although it was increased in the PVN on day 2. Significant hypertrophy of ir-OXT neurons was observed after the second day of the sodium loading (Figs. 3 and 7). The temporal pattern of changes in magnitudes of hypertrophy that was found in supraoptic and paraventricular OXT neurons was similar to that observed in paraventricular AVP neurons (Fig. 7).

Neurohypophysis

Although immunoreactivity of the neurohypophysis was not analyzed quantitatively, it appeared that AVP and OXT immunoreactivities were markedly decreased after day 4 and day 7 of the sodium loading, respectively, indicating a de-

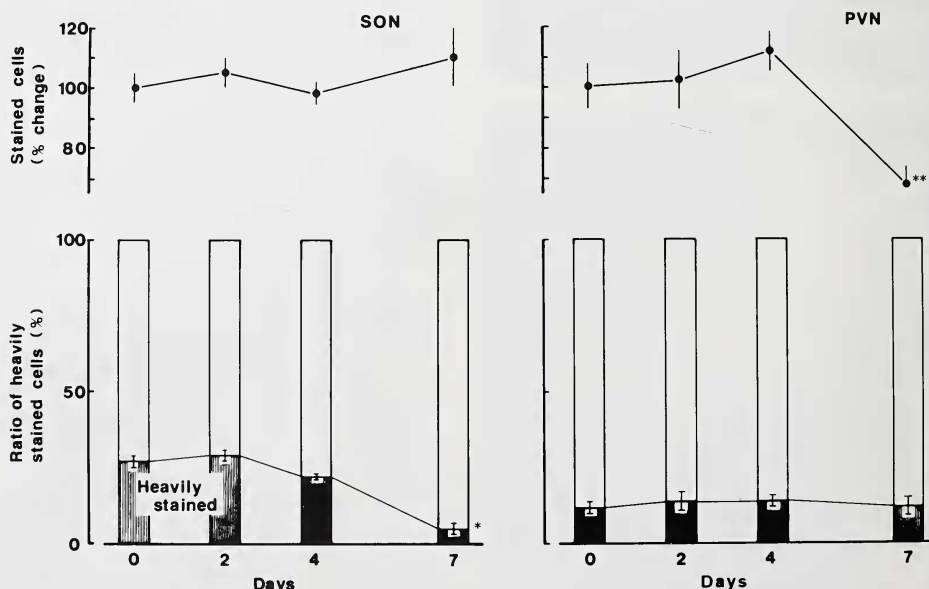


Fig. 5. Effects of sodium loading on the number of stained AVP neurons (percent change) compared to day 0 (the numbers per section are: SON, 47.0 ± 2.5 ; PVN, 54.8 ± 4.3), and the percentage of heavily stained AVP neurons per total stained AVP neurons in the SON and the PVN. Each point represents the mean \pm S.E. ($n=7$). *, $p < 0.05$; **, $p < 0.01$; by the t-test compared to day 0.

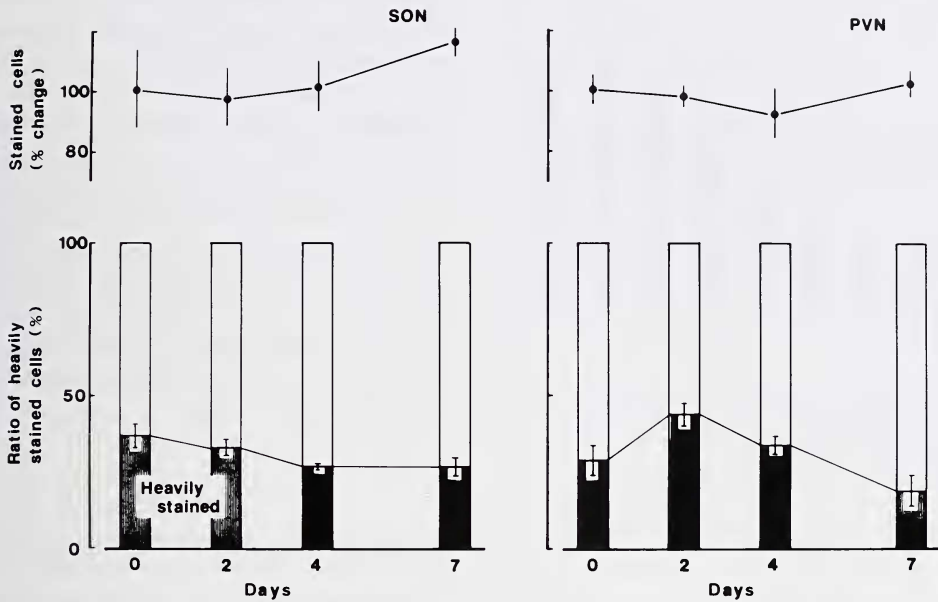


FIG. 6. Effects of sodium loading on the number of stained OXT neurons (percent change) compared to day 0 (the numbers per section are: SON, 45.1 ± 6.9 ; PVN, 72.8 ± 3.9), and the percentage of heavily stained OXT neurons per total stained OXT neurons in the SON and the PVN. Each point represents the mean \pm S.E. ($n=7$).

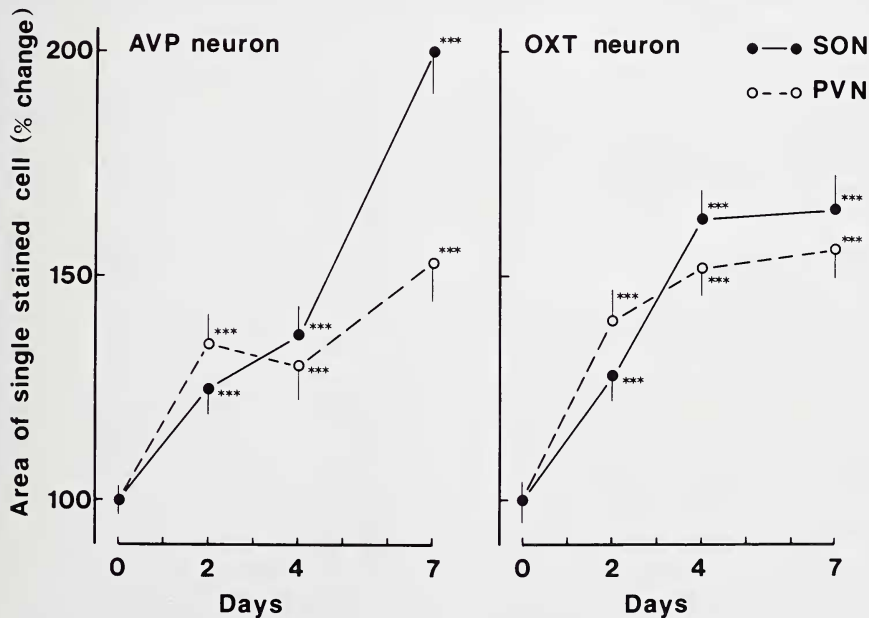


FIG. 7. Effects of sodium loading on sizes of the AVP and OXT immunoreactive neurons in the SON and the PVN. The values of cell areas ($\times 10^{-4} \text{ mm}^2$) on day 0 are: AVP neurons in the SON, 2.54 ± 0.08 ; AVP neurons in the PVN, 3.25 ± 0.11 ; OXT neurons in the SON, 2.57 ± 0.12 ; OXT neurons in the PVN, 2.34 ± 0.09 . Each point represents the mean \pm S.E. ($n=7$). ***, $p < 0.001$; by the t-test compared to day 0.

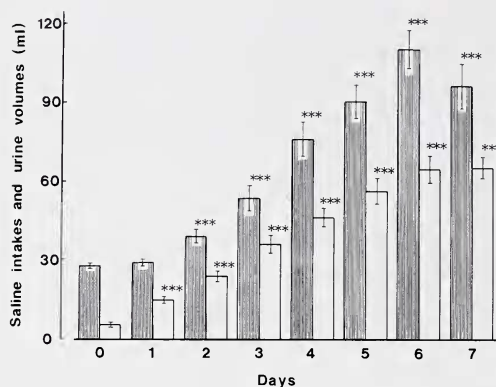


FIG. 8. Changes in oral intake (hatched bars) and urine volume (open bars) by oral hypertonic saline. Each point represents the mean \pm S.E. ($n=7$). ***, $p < 0.001$; by the t-test compared to day 0.

pletion of stored pools of neurohypophysial hormones.

Changes in plasma and urine by oral hypertonic saline

The oral intake of 2% hypertonic saline was progressively increased during the experimental

period, and attained to the maximum equilibrium rate by day 6. The urine volumes were similarly increased (Fig. 8). The plasma osmolality and the plasma Na^+ concentration were elevated to the significant level within 2 days after the onset of oral hypertonic saline, and were further increased linearly at the same rate (Fig. 9). The urine Na^+ concentration was also significantly increased after day 2 of the treatment. On the contrary, the urine osmolality was drastically decreased after day 2 of the treatment (Fig. 9). These results indicate that the animals under the oral hypertonic saline regimen were exposed to an osmotic stimulus with hypertonic plasma sodium.

DISCUSSION

The present study showed that the hybridization signals for the AVP and OXT mRNAs were increased in the SON and the PVN by oral hypertonic saline, indicating that the AVP and OXT mRNA levels in magnocellular neurons were elevated by hypertonic sodium stimulation. This result implies that the sodium loading stimulated

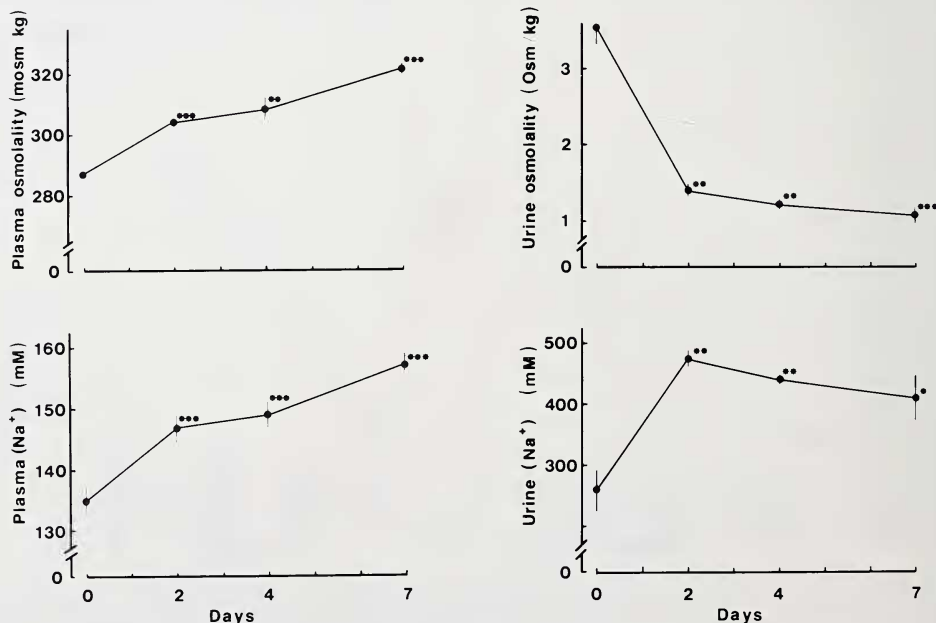


FIG. 9. Changes in plasma osmolality, plasma $[\text{Na}^+]$, urine osmolality and urine $[\text{Na}^+]$ by oral hypertonic saline. Each point represents the mean \pm S.E. ($n=7$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; by the t-test compared to day 0.

transcription of the genes encoding neurohypophyseal hormones in both the SON and the PVN. In addition, the sodium loading induced significant hypertrophy of both AVP and OXT neurons. This fact indicates that translation rates of AVP and OXT precursors were also increased by the sodium loading, since chronic dehydration elicited several changes which indicate stimulation of protein syntheses in magnocellular neurosecretory neurons, that is, an increase in amino acid incorporation [5, 6], increases in nuclear and nucleolar diameters and dilation of endoplasmic reticulum [7]. Nevertheless, stainability of ir-AVP neurons was reduced in both the SON and the PVN, probably because of a rapid transport of newly synthesized AVP to the neurohypophysis, as was discussed in the previous paper [11].

Although the increase in the AVP mRNA level was observed in both the SON and the PVN, it was more rapid and marked in supraoptic AVP neurons. This result agrees with the report of Sherman *et al.* [15]. In the present study, the hypertrophy of AVP neurons was also more marked in the SON than in the PVN. Similar differences in response to sodium loading have been observed between the SON and the PVN by several investigators. A significant increase in incorporation of cytidine into RNA [22] and that of tyrosine into peptides [6] in response to oral hypertonic saline were demonstrated in the SON, but not in the PVN. Activation of the electrical activity of paraventricular AVP neurons in response to an increase in plasma osmolality was much less than that of supraoptic AVP neurons [3]. Furthermore, the possibility that supraoptic neurons are directly osmosensitive and play a part of an osmoreceptive complex has been indicated [23, 24]. These results suggest that AVP neurons in the SON are more responsible for osmotic regulation than paraventricular AVP neurons.

The OXT mRNA was increased by the sodium loading. Marked hypertrophy was also observed in ir-OXT neurons within 2 days after the onset of the treatment. These results may imply that OXT neurons have some physiological role in the water and salt metabolism. This possibility is supported by the facts that OXT release was stimulated by sodium loading, and that OXT has been implicated

in the control of renal functions [2]. In addition, the increase in the OXT mRNA was more rapid than that in the AVP mRNA. Since OXT neurons released more products than AVP neurons in response to an acute increase in plasma osmolality [4], OXT neurons may be more sensitive to an acute increase in plasma osmolality than AVP neurons.

A rise in plasma osmolality immediately increased the firing rates of AVP and OXT neurons in both the SON and the PVN, and then plasma AVP and OXT levels were rapidly increased [3]. On the contrary, the present study showed that statistically significant increases in the AVP and OXT mRNA levels were detectable after 4- and 2-day sodium loading, respectively. The reason for this difference in the onsets of secretory activity and synthetic activity is not clear at present. It may reflect the presence of abundant storages of these hormones in the neurohypophysis and/or the hypothalamic nuclei. Further investigations are required to clarify signals by which the onset of gene expression is stimulated.

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Goitrogenic Action of Manganese on Female Mouse Thyroid through Three Generations

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ABSTRACT—The effect of manganese on mouse thyroids was examined through three generations. The continuous supply of drinking water containing 200 mg/l $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ induced mild goiter only in female mice.

The manganese treatment did not significantly affect the serum thyroxine (T_4) levels in dams or the plasma levels in neonates, suggesting that the effect was not severe. The syntheses of T_4 and triiodothyronine (T_3) were examined in hydrolyzed thyroids. The results showed that ratios of radioiodide activity in hormonal fractions of the manganese-given groups to those of control groups were greater than 1 for all three generations of female mice. These results indicated that newly synthesized hormones were retained in thyroids of the manganese-treated female mice. On the other hand, in males, the ratio was reduced to less than 1 as the numbers of generations increased.

"Dwarf" neonates born in the third generation of the manganese-treated group showed an ataxial motion in their gait of walk. However, histological examination of cerebral and cerebellar regions of the dwarfs revealed no severe changes.

INTRODUCTION

It has been shown that excess manganese induces an endemic goiter when iodide intake is low [1, 2]. In laboratory experiments, acute and sub-acute administrations of manganese suppressed thyroidal iodide uptake and affected iodide metabolism [3, 4]. The authors also demonstrated that the radioactive manganese accumulated to a high level in mouse thyroid as well as in other endocrine organs, such as the pituitary, pancreas and adrenal [5]. Furthermore, it was found that under ordinary iodide intake, a 7 week administration of manganese in the drinking water resulted in thyroid enlargement in female mice, but not in male mice [5].

For goiter formation, a prolonged manganese treatment through an oral route was necessary because a single [3, 6] or a sustained [4] parenteral injection of manganese caused very severe reduc-

tions of iodide uptake and synthesis of thyroid hormones, but did not induce thyroid enlargement. Although prolonged treatment increases thyroid hormone levels in the gland and reduced the hormones in the blood [5], the mechanism of goiter formation by manganese is not well understood. Thus, we were interested in determining whether oral administration of manganese for several generations caused larger goiters, and also whether hereditary factors were involved in the manganese-induced goiter. In this study, we examined the chronic effects of manganese on growth, thyroid function and brains of mice in three consecutive generations.

MATERIALS AND METHODS

Chemicals The chemical reagents used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan) and oxytocin was from Sigma Chemical Co. (St. Louis, MO., USA). [^{125}I]Na was purchased from New England Nu-

clear Co. (Boston, MA., USA). The radioimmunoassay kit, SPAK T₄ was purchased from Daiichi Isotope Institute (Tokyo, Japan). Pronase was a product of Kaken Pharmaceutical Co. (Tokyo, Japan), and filter paper No. 51 for chromatography was obtained from Toyo Roshi Co. (Tokyo, Japan).

Animals Male and female ddY mice weighing 18–20 g (4 weeks old) were obtained from a local supplier and maintained on a 12 hr light-dark cycle in an air conditioned room at 23–24°C and 50% moisture. Animals were allowed free access to solid food (Type MF, Oriental Yeast Co. Tokyo, Japan) and tap water for the control. The iodide and manganese contents in the food pellets were 1.04 and 64.5 µg/g, respectively, as reported previously [5]. Mice were given 200 mg/l of MnCl₂·4H₂O in the drinking water for 7 weeks. These animals were designated as the first generation of manganese-treated mice (parents). Some mice were sacrificed at 11 weeks of age to examine thyroid enlargement. The remaining mice were mated to provide the second generation of manganese-treated mice. During mating and subsequent pregnancy, manganese administration was continued. The offsprings from these parents with manganese treatment were designated as the second generation. The third generation of manganese-treated mice was obtained by a brother-sister mating of the second generation with manganese treatment.

On day 1 when neonates were born, they were anesthetized with ether and blood was collected into a heparinized test tube by cutting the carotid artery. The blood from a litter was pooled and centrifuged to obtain plasma for hormone analysis. Another group of neonates was sacrificed on day 5 to collect plasma. Dams were also sacrificed on day 1 and day 5 after delivery. Blood samples were taken from the dams. The thyroids were excised and their weights were recorded.

Intrathyroidal iodine metabolism Mice treated with manganese and the control were intraperitoneally given 3–4 µCi [¹²⁵I]Na per animal 24 hr prior to sacrifice. At autopsy, the thyroids were excised, weighed and pooled in each group because an individual thyroid was insufficient for analysis. The thyroids were added to a test tube

containing 50 mM phosphate buffer (pH 7.4), 1 mM methylmercaptoimidazole and two drops of ethanol. The mixture was subjected to proteolytic hydrolysis with Pronase (20:1 weight ratio). For this purpose, the test tube was bubbled with N₂ gas, sealed with a stopper and incubated at 37°C for 24 hr. Digestion was terminated by immersing the tube in boiling water. Samples of the hydrolysates were spotted on paper and subjected to paper chromatography using two different solvent systems (*n*-butanol : acetic acid : H₂O = 4:1:2, v/v and *n*-butanol : ethanol : 2N ammonium hydroxide = 5:1:2, v/v). Radioiodinated compounds were analyzed by autoradiography. Areas on the paper showing dark spots were cut and their radioactivities were measured by an auto gamma scintillation counter.

Hormone measurement Serum was prepared from adult animals, but pooled plasma was prepared from neonates because of an insufficient volume of blood from an individual neonate. Circulating T₄ was assayed by SPAK T₄ RIA kit. In this assay system, 25 µl of specimen were used in a single measurement and at least duplicate measurements were carried out for each specimen. A cross reaction with T₃ was not detectable with this kit.

Histological study Thyroids were excised and fixed in Bouin's fluid. Using ordinary processes, samples were embedded in a paraffin wax, sliced and sections were stained in hematoxylin-periodate. Whole brain regions were fixed in 10% formaldehyde solution and embedded in an epon. The specimens were stained in luxol fast blue-cresylechtviolet solution for simultaneous staining of Nissl bodies and the myelin sheath, and in hematoxylin-eosin solution for ordinary staining. Stainability and histological changes were examined under a light microscope.

RESULTS

Thyroid enlargement by manganese

A morphological study of thyroids of female mice treated with 200 mg/l manganese chloride for 7 weeks revealed moderate size goiters with colloid filled lumens and flattened epithelial cells. Table 1

TABLE 1. Manganese effect on body and thyroid weights in three generations of mice

	1st generation		2nd generation		3rd generation	
	C	Mn	C	Mn	C	Mn
Female						
No. of animal	14	12	11	10	9	9
Body weight (g)	31.0±3.9	32.5±2.5	32.0±3.0	32.3±2.4	32.0±3.9	31.2±2.5
Thyroid weight (mg)	2.7±0.3	3.3±0.3	3.1±0.3	4.3±0.4	2.8±0.5	3.7±0.4
	p<0.001		p<0.001		p<0.001	
Male						
No. of animal	9	11	16	11	11	11
Body weight (g)	40.6±3.4	40.9±3.0	42.2±4.1	43.2±3.8	41.5±3.4	44.3±3.4
Thyroid weight (mg)	3.3±0.4	3.8±1.0	3.5±0.4	3.8±0.3	3.6±0.3	3.6±0.3
	NS		NS		NS	

C: Control, Mn: Mn treated, NS: Not significant.

shows that, in the first generation, thyroids of female mice were enlarged by manganese chloride, but thyroids of male mice were unchanged by the same treatment. When the mice with manganese treatment were mated, 9 out of 15 females became pregnant (60%), whereas in the control group 8 out of 12 became pregnant (67%). In the second generation, the frequencies of pregnancy of the manganese-treated female mice and the control were 7/14 and 10/15, respectively. The body weight gain and other external appearances were not changed by manganese treatment. At delivery time, the average number of neonates bred and their mean body weight were also similar to those of control group. When animals of the second generation were 11-12 weeks old, some were sacrificed to examine their thyroids. The thyroid weight of the females of the second generation was slightly greater than that of the first generation. In the third generation, the size of glands was similar to that of the first generation but smaller than that of the second generation.

Manganese effect on circulating T_4 level

The results of the circulating T_4 levels of non-pregnant adult female mice, dams after delivery (day 1 and day 5) and neonates (day 1 and day 5)

are summarized in Table 2. Just after delivery, serum T_4 levels of dams were within normal ranges and not influenced by the birthing process. Dams with manganese treatment maintained normal T_4 levels at day 1. However, the T_4 levels of dams in both groups fell to subnormal levels during the term of lactation (day 5). T_4 levels of neonates on day 1 were very low but increased slightly during the first five days. This increase in T_4 level was also observed in the manganese-treated group. Thus, there was no significant effect of manganese on circulating T_4 levels.

Manganese effect on radioiodide uptake into hormonal fraction of thyroid

In order to determine whether manganese interferes with hormone synthesis, the radioiodide uptake into intrathyroidal hormonal fractions was examined in control and manganese-treated mice through three generations. Table 3 shows the radioiodide distribution in T_3+T_4 fractions of each group. ^{125}I activity in the females treated with manganese was much higher (10-20%) than that of the control group, resulting in the ratios of Mn/C being greater than one or at least equal to one. On the other hand, in males, the ratios of Mn/C were always less than one. Furthermore, as

TABLE 2. Manganese effect on thyroxine level in blood

Group		1st generation	2nd generation	3rd generation
		ng/ml	ng/ml	ng/ml
Non-pregnant adult female	Control	38.5±5.0 (n=4)	38.9±7.9 (n=4)	
	Mn-treated	33.5±2.5 (n=4)	40.8±8.5 (n=5)	
Dam	Control	38.0±4.7 (n=6)	28.1±1.9 (n=4)	
	Mn-treated	37.5±14.2 (n=5)	30.8±7.6 (n=6)	
	Control	17.6±4.2 (n=6)	16.8±1.0 (n=4)	
	Mn-treated	21.8±8.7 (n=6)	16.5±1.6 (n=6)	
Neonate	Control		6.4±6.3 (n=4)	9.4±3.9 (n=4)
	Mn-treated		5.1±2.9 (n=5)	14.1±5.9 (n=6)
	Control		10.3±0.6 (n=3)	14.9±1.9 (n=4)
	Mn-treated		18.1±0.1 (n=2)	17.9±1.6 (n=6)
	Control			
	Mn-treated			

Data show serum hormone levels for adults and plasma hormone levels for neonates. Each value is the mean±SD.

The numbers within the parentheses for the adult females and dams represent the numbers of mice used in the study. However, the numbers within the parentheses for neonates represent the numbers of litters in each group.

the number of generations increased, the ratios became smaller in male mice.

"Dwarfs" and the histological examination of their brains

Continuous manganese administration did not affect the growth of parents and the second generation of mice when their body weights were monitored. However, in the third generation of the manganese-treated group, some smaller neonates were observed, although the incidence was not high: 0/11 for the control and 3/17 for the manganese-treated group (The numerator and denominator represents numbers of dams which bred

a "dwarf" and total numbers of dams examined, respectively. In this experiment, the term "dwarf" is defined for neonates weighing less than 70% of the average body weight of siblings in the same litter on day 1). After a half number of normal siblings were isolated from their mother, dwarfs were still unable to grow at the rate of normal mice, suggesting that the dwarfs were not the results of interrupted lactation but of some other endogenous dysfunctions.

Figures 1 and 2 illustrate the growth curves and the photograph of normal and dwarf mice, respectively. Dwarfs grew slowly and never reached the level of body weight of normal animals. Since